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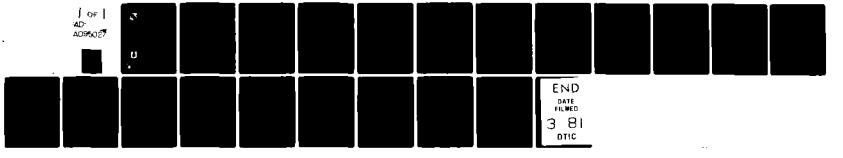

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FJSRL TECHNICAL REPORT 80-006

DECEMBER 1980

THE EFFECT OF HYPERBARIC OXYGEN ON THE GROWTH OF RHIZOPUS NIGRICANS

BY

WILLIAM J. CAIRNEY

KAREN A. O'HAIR

PROJECT 2303

AIR FORCE SYSTEMS COMMAND

UNITED STATES AIR FORCE

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Hyperbarics	Oxygen Pressure	

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Plus (+) and minus (-) mating strains of Rhizopus nigricans were observed in vitro for macroscopic and microscopic growth characteristics at varying hyperbaric oxygen levels and for various exposure times and intervals. Definite inhibition of both strains was observed within oxygen levels well tolerated by humans. Overall experimental results provide in vitro basis for use of compression chamber therapy in treatment of mucormycosis caused by Rhizopus nigricans.

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THE EFFECT OF HYPERBARIC OXYGEN ON THE GROWTH OF *RHIZOPUS NIGRICANS*

BY

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DECEMBER 1980

DEPARTMENT OF BIOLOGY
U. S. AIR FORCE ACADEMY, COLORADO 80840

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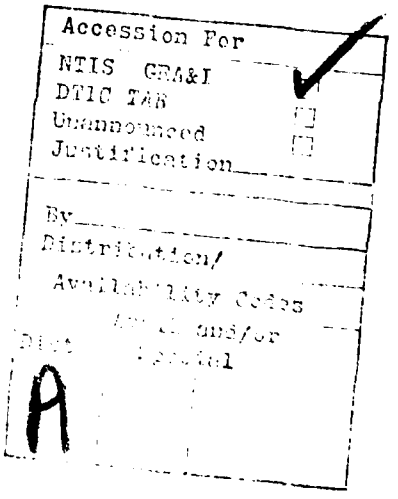

INTRODUCTION

Hyperbaric oxygen has been used effectively for almost two decades for treatment of anaerobic infections (e.g. clostridial myonecrosis) and carbon monoxide poisoning. In the late 1960's, reputable research facilities began reporting clinical successes in the use of hyperbaric oxygen for treatment of Pseudomonas infections secondary to burns, osteogenesis enhancement in bone grafts, osteomyelitis, maxillofacial osteoradionecrosis, and preservation of failing skin grafts (7). Successful treatment of infections caused by aerobic organisms including several fungi has prompted some recent research efforts to further investigate the effect of hyperbaric oxygen on mycotic disease agents. Cairney has reported the effects of hyperbaric oxygen on Aspergillus fumigatus, Candida albicans, several species of dermatophytes, and Mucor sp. (2,3,4,5).

Treatment of mucomycotic disease agents with hyperbaric oxygen has attracted special interest. Two cases of mucormycosis have been treated at the Hyperbaric Medicine Division, USAF School of Aerospace Medicine. Hyperbaric oxygen was used as a last resort treatment in both cases, but in each case the mycosis cleared (6). Baker reports that the predominant organism isolated from patients with mucormycosis is Rhizopus, not Mucor. Rhizopus oryzae, R. arrhizus, R. nigricans, R. rhizopodiformis, and R. microsporus have all been cultured from human subjects (1).

Perhaps the most extensive study of the reactions of fungi to hyperbaric oxygen was carried out by Robb (8). Robb exposed 103 species of fungi to 10 atmospheres oxygen pressure at 25°C for 7 days. This exposure is well in excess of established human oxygen tolerance limits. There are no reports in the literature of the reaction of Rhizopus spp. to hyperbaric oxygen at PO_2 levels not toxic to humans.

The purpose of this study was to determine the growth rate and observe the macroscopic and microscopic appearance of Rhizopus nigricans grown in culture under various hyperbaric oxygen levels.

MATERIALS AND METHODS

Three experiments were undertaken to determine the effects of hyperbaric oxygen on Rhizopus nigricans. Isolates of the fungus (+ and - mating strains) were obtained from the American Type Culture Collection. Potato dextrose agar (PDA) was used as a culture medium in all experiments. All exposures of the organisms to increased oxygen were carried out in a table top (31 liter capacity) hyperbaric chamber made available for the project by the Aerospace Pathology Branch of the Armed Forces Institute of Pathology.

In all experiments, organisms were compressed to the desired pressure in less than 30 seconds. Following compression, the chamber was flushed rapidly with five chamber volumes of 100% oxygen to insure a pure oxygen environment. Each time the chamber was decompressed for removal of cultures, this same flushing operation was performed upon recompression. During compression periods, a 5-liter/hour flow was maintained through the system.

Organisms to be exposed to oxygen, along with controls, were propagated from growing hyphal tips. In the first two experiments agar biplates were used. The two compartments contained the + and - strains, respectively. In the last experiment, each undivided plate contained a + and - strain of Rhizopus nigricans. No inhibitors of any kind were incorporated into the medium in order to keep variables to a minimum. The pH of the medium was 5.6. The room in which the experiments were performed was maintained at 25° (±1°) C. Cultures were exposed to external fluorescent light for the duration of all experiments. The hyperbaric chamber, constructed of transparent plexiglass, allowed exposures of organisms to external light.

In the first experiment, + and - strains of Rhizopus nigricans were placed in the hyperbaric chamber and exposed to a steady 1-ATA (one atmosphere absolute) level of oxygen. Controls were allowed to grow at room pressure. Three 1-ATA air control plates (12 potential colonies) and three 1-ATA level of oxygen plates (12 potential colonies) were used for the first experiment. To insure growth, all colonies were allowed to incubate 48 hours before placing the 100% oxygen plates into the chamber. All plates were surveyed visually for macroscopic growth at 24-hour intervals for three days. Two additional readings were taken on the 11th and 18th day after inoculation.

In the second experiment, Rhizopus nigricans cultures were exposed to hyperbaric oxygen according to standard exposure tables used for treating gas gangrene resulting from Clostridium spp. (especially Clostridium perfringens) infections (3,4). As in the first experiment, Petri dishes containing 24-hour cultures of + and - Rhizopus nigricans were used. Three plates (12 potential colonies) were used for chamber exposure. Three plates (12 potential colonies) were used as 1-ATA air controls.

The experimental plates were exposed to 3-ATA oxygen for three 90-minute periods during the first 24 hours. In each of two succeeding 24-hour periods, cultures were exposed to the same 3-ATA level for two 90-minute periods. Cultures were thus exposed to seven 90-minute periods of oxygen at 3-ATA over a span of three days. Organisms were checked for growth throughout the experiment and measurements were made on the first, third, sixth, and eighth days after inoculation.

In the third experiment, Rhizopus nigricans cultures were also exposed to hyperbaric oxygen according to exposure tables used for treating gas gangrene, but exposure of some plates was continued for two 90-minute

periods per day for up to two additional days. Cultures were thus exposed to seven, nine, or eleven 90-minute periods of oxygen at 3-ATA over a span of three, four, or five days, respectively. Undivided plates were used. One colony of each strain (+ and -) were placed together on each Petri dish. Ten plates (20 potential colonies) were exposed to the standard treatment table. Six plates (12 potential colonies) followed the standard exposure treatment table plus two 90-minute exposures during an additional 24 hours. Five plates (10 potential colonies) were exposed to the standard treatment table then given four additional 90-minute exposures, two exposures during each of the next 24-hour periods. Six plates (12 potential colonies) were maintained as 1-ATA air controls.

Plates to be exposed were placed in the chamber 24 hours after inoculation. Measurements of the controls and the standard treatment table plates began on the fourth day after inoculation. The other plates were measured on the day their scheduled exposure were completed. All measurements of the colonies were taken until the eleventh day after inoculation.

In each of the three experiments air pressurized controls (3-ATA) were run at a separate time but under otherwise identical conditions to insure that any observed effects were caused by oxygen rather than by total pressure. In each case, the number of compressed air control plates equaled the number of plates exposed to pressurized oxygen. Exposure to 3-ATA compressed air resulted in no significant differences in growth rates between exposure plates and 1-ATA air controls.

RESULTS

Growth curves for all organisms in the 1-ATA experiment are shown in figures 1 and 2. Growth curves for the first "treatment table" experiment are shown in figures 3 and 4. Growth curves for the second

"treatment table" exposures are shown in figures 5 and 6. Fungi exposed to identical conditions (whether on the same actual Petri plate or not) grew to the same extent over a given time interval. An upper limit was established for the diameter of the colony, 50mm. Once a colony had reached this size no more readings were taken. This upper limit was mandated by the size of the Petri plate.

Exposure to 1-ATA oxygen resulted in no significant differences in growth rates between exposed plates and controls. However, there is a marked difference between the growth rates of + and - strains overall. The - strain of Rhizopus nigricans grew at a much faster rate, always reaching 50mm in size. The + strain never reached a 50mm diameter size in either chamber or control plates. This fact was evident in not only the 1-ATA experiment but also in the other experiments.

Figures 3 through 6, from the second experiment, show that the - Rhizopus nigricans growth, while not inhibited altogether, is at least retarded. The exposed + Rhizopus nigricans, however, grew at a faster apparent rate than the controls. One major reason for this was the starting size of the R. nigricans + colonies when placed in the chamber. The plates destined for the chamber were specifically picked because of the size of the + strain colonies. Due to the somewhat less dependable growth characteristics of the R. nigricans + strain, vigorus growth had to be assured before the organism was exposed to hyperbaric oxygen. An admitted bias was therefore present in this experiment in favor of the + strain. One significant developmental feature was that sporangial growth was delayed in all exposed colonies compared to the controls for the R. nigricans - strain. The R. nigricans + strain never showed any sporangial growth during any of the experiments.

The third experiment results seemed to partially contradict the results from the second experiment. The plates exposed to the standard treatment table showed no significant difference between their growth rate for both the + and - Rhizopus nigricans and the controls. Sporangia, furthermore, were formed after the same interval after inoculation in both the standard treatment exposures and the controls. However, as the number of days of exposure increased, the difference between the growth rate of the exposed organisms to the control organisms increased. Also, the delay in formation of sporangia was observed to increase with the number of additional days of exposure.

One observation, however, was consistent in the 3-ATA hyperbaric oxygen experiments. The density of growth was much less for fungi on the exposed plates than on the controls while the former were undergoing hyperbaric oxygen treatment. The hyperbaric oxygen-treated colonies were sparsely matted and were tending to grow into agar rather than on the surface. The controls were thickly matted and largely growing on the surface. After the exposed colonies were removed from hyperbaric oxygen, however, they started growing on the surface and quickly became thickly matted.

DISCUSSION

The intent of this investigation is to establish an in vitro cytological basis for treating cases of mucormycosis in hyperbaric chambers. To some extent, that basis has been established. The growth of Rhizopus nigricans was at least retarded by oxygen levels well tolerated by humans.

The 1-ATA oxygen exposure, had it had an effect, would have at least suggested the possibility of treatment with 100% oxygen without the need for additional pressurization.

The demonstration of an effect using a standard treatment table is significant and the effect of the hyperbaric oxygen might be greatly enhanced with systemic antifungal agents used between oxygen exposures. Unpublished data by Simon (9) suggest that some antifungal agents, including Amphotericin B, may actually be degraded by exposure to hyperbaric oxygen. One or more of the breakdown products may be metabolites. Should this be confirmed in future work, combining hyperbaric oxygen with systemic antifungal agents administered at the same time might not be advisable.

Follow-on *in vivo* hyperbaric oxygenation studies testing mucormycotic agents, especially *Rhizopus* spp. would supply additional helpful information. For future *in vitro* work, Elliott (this laboratory) is developing a highly precise liquid culture technique for assessing the developmental effects of hyperbaric oxygen on human pathogenic fungi in culture.

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The authors gratefully acknowledge the support of Dr. Gary R. Coulter, Associate Professor of Biology, Dr. Lawrence J. Biever, Assistant Professor of Biology, both of the USAF Academy, and Dr. Kenneth E. Siegenthaler, Director of Chemical Sciences, Frank J. Seiler Research Laboratory, for review of the manuscript and technical advice. The support of Mr. John Scupp and Mr. Michael Petraglia was invaluable for providing laboratory assistance. The authors also appreciate the support of the Frank J. Seiler Research Laboratory in sponsoring this effort.

Note: Reference cultures of the + and - mating strains of *Rhizopus nigricans* used in this study are maintained under oil in the Biology Department (DFB) Culture Collection, USAF Academy, Colorado.

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FIGURE 1

GROWTH CURVES FOR CULTURES OF RHIZOPUS NIGRICANS USED AS CONTROLS IN EXPERIMENT 1

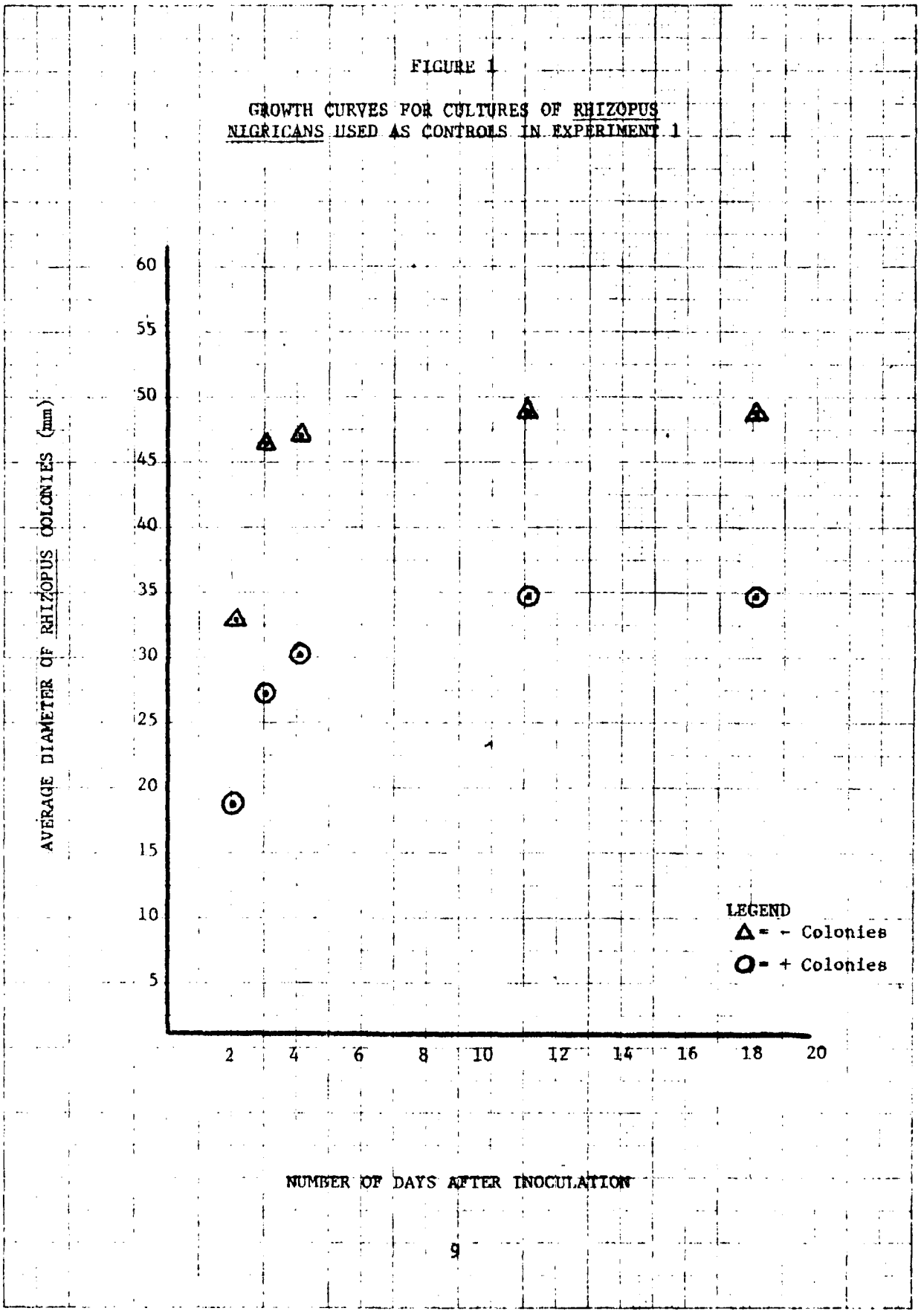


FIGURE 2

GROWTH CURVES FOR RHIZOPUS NIGRICANS CULTURES EXPOSED TO 100% O_2 AT 1-ATA

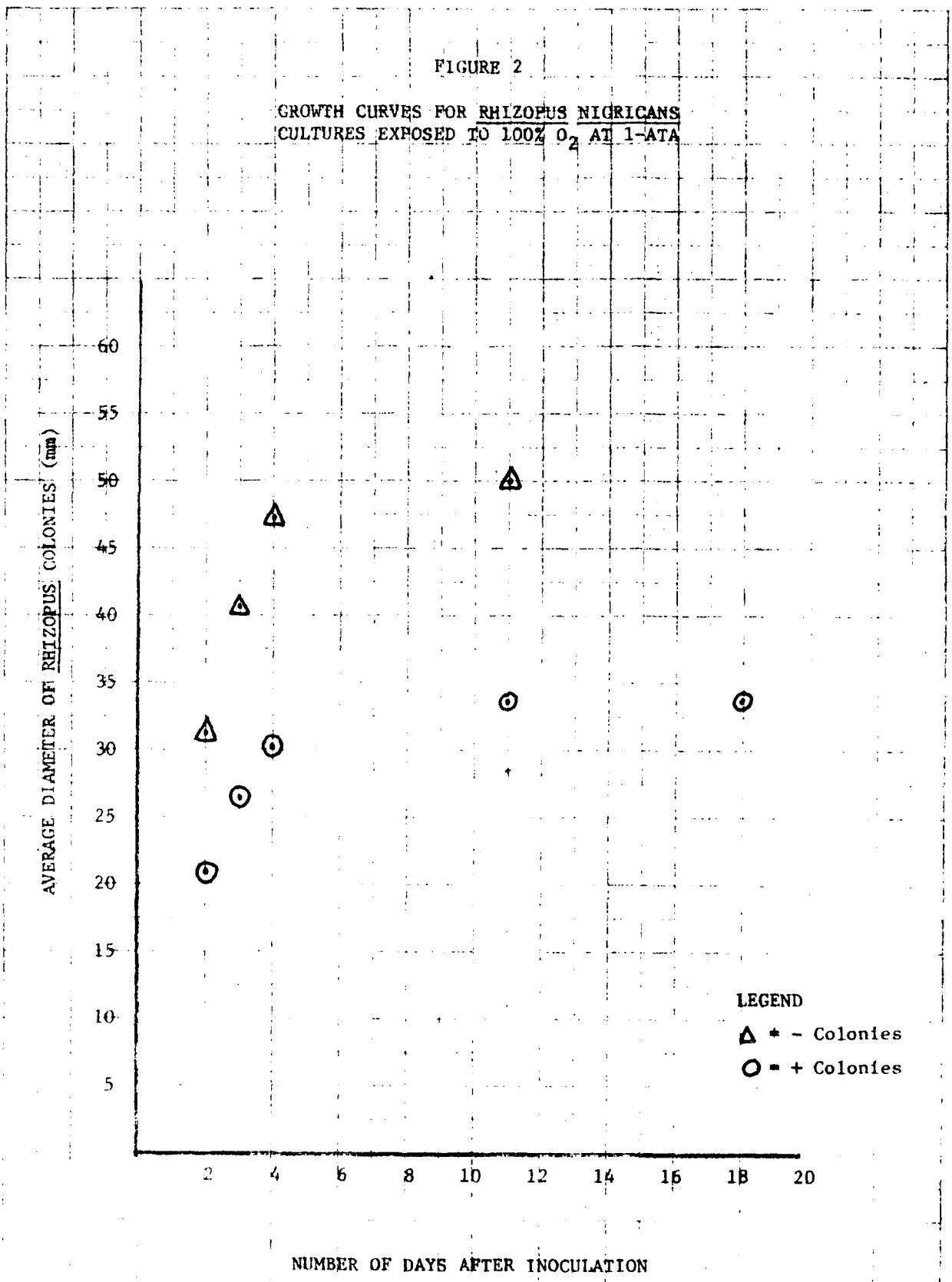


FIGURE 3

GROWTH CURVES FOR RHIZOPUS NIGRICANS CULTURES
USED AS CONTROLS FOR "STANDARD TREATMENT TABLE"
EXPERIMENT (EXPERIMENT 2, RUN #1)

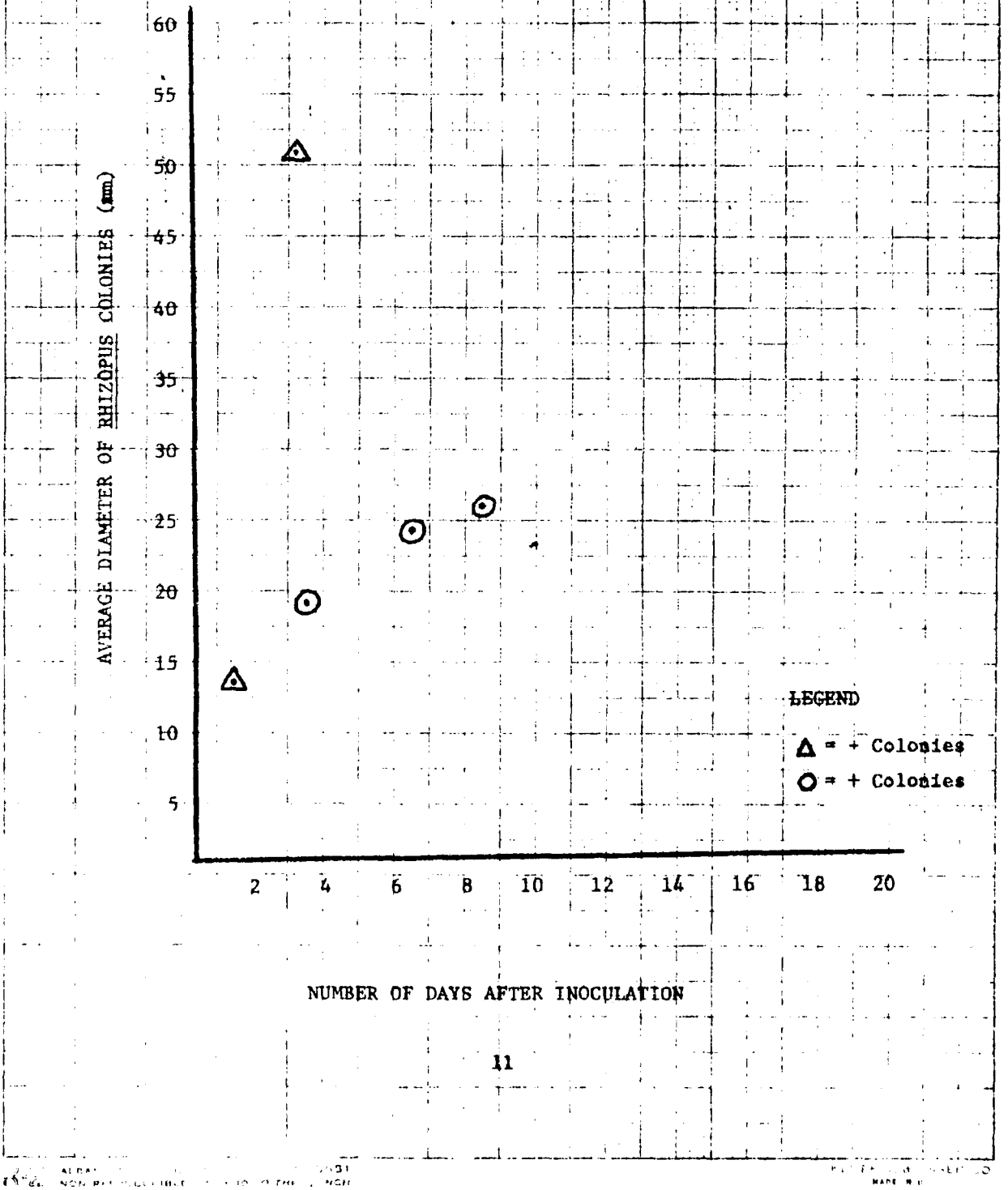


FIGURE 4

GROWTH CURVES FOR RHIZOPUS NIGRICANS CULTURES SUBJECTED TO "STANDARD TREATMENT TABLE" OXYGEN EXPOSURES (EXPERIMENT 2, RUN #1)

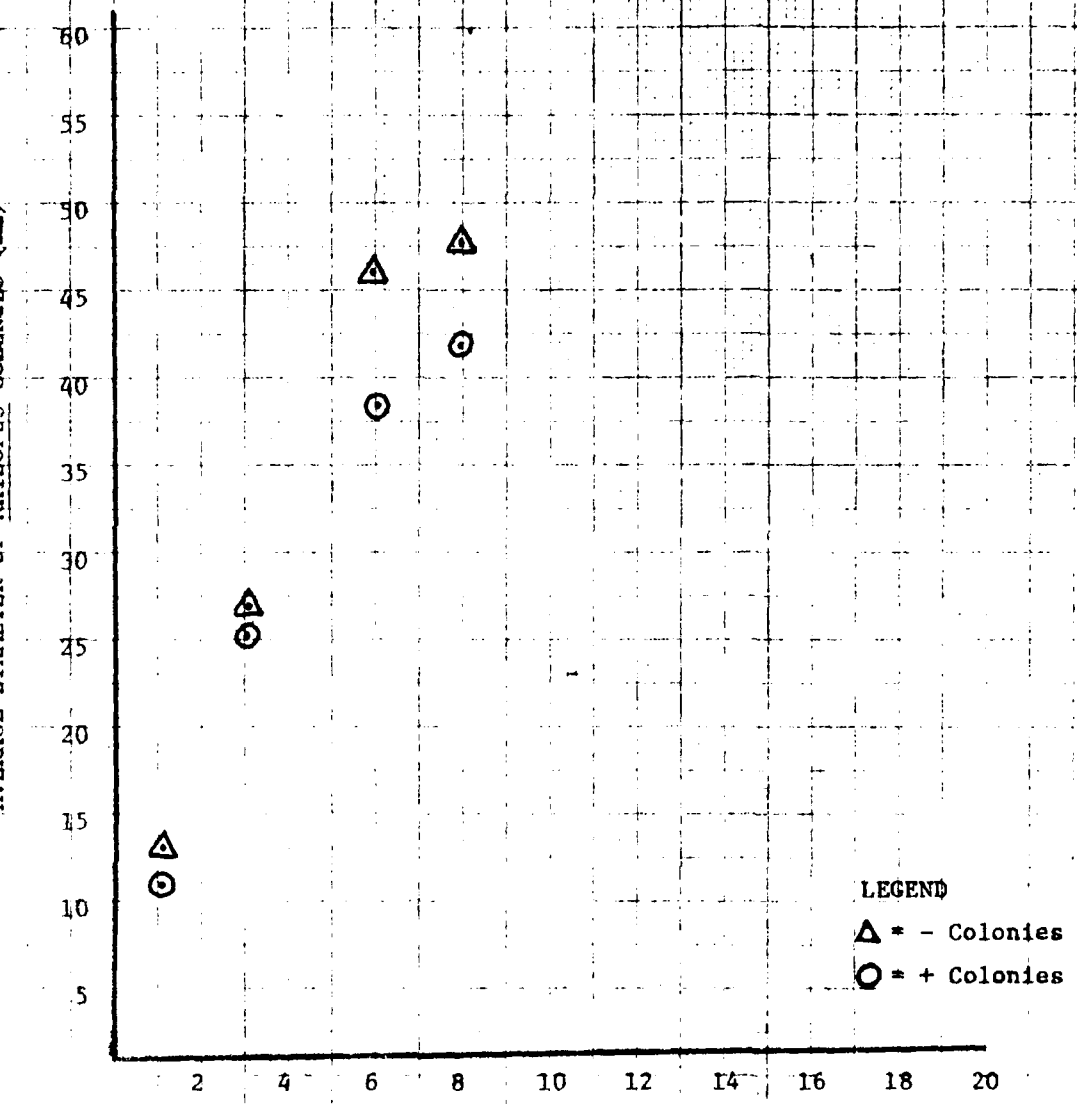


FIGURE 5

GROWTH CURVES FOR RHIZOPUS NIGRICANS CULTURES USED AS CONTROLS FOR "STANDARD TREATMENT TABLE" EXPERIMENT (EXPERIMENT 2, RUN #2)

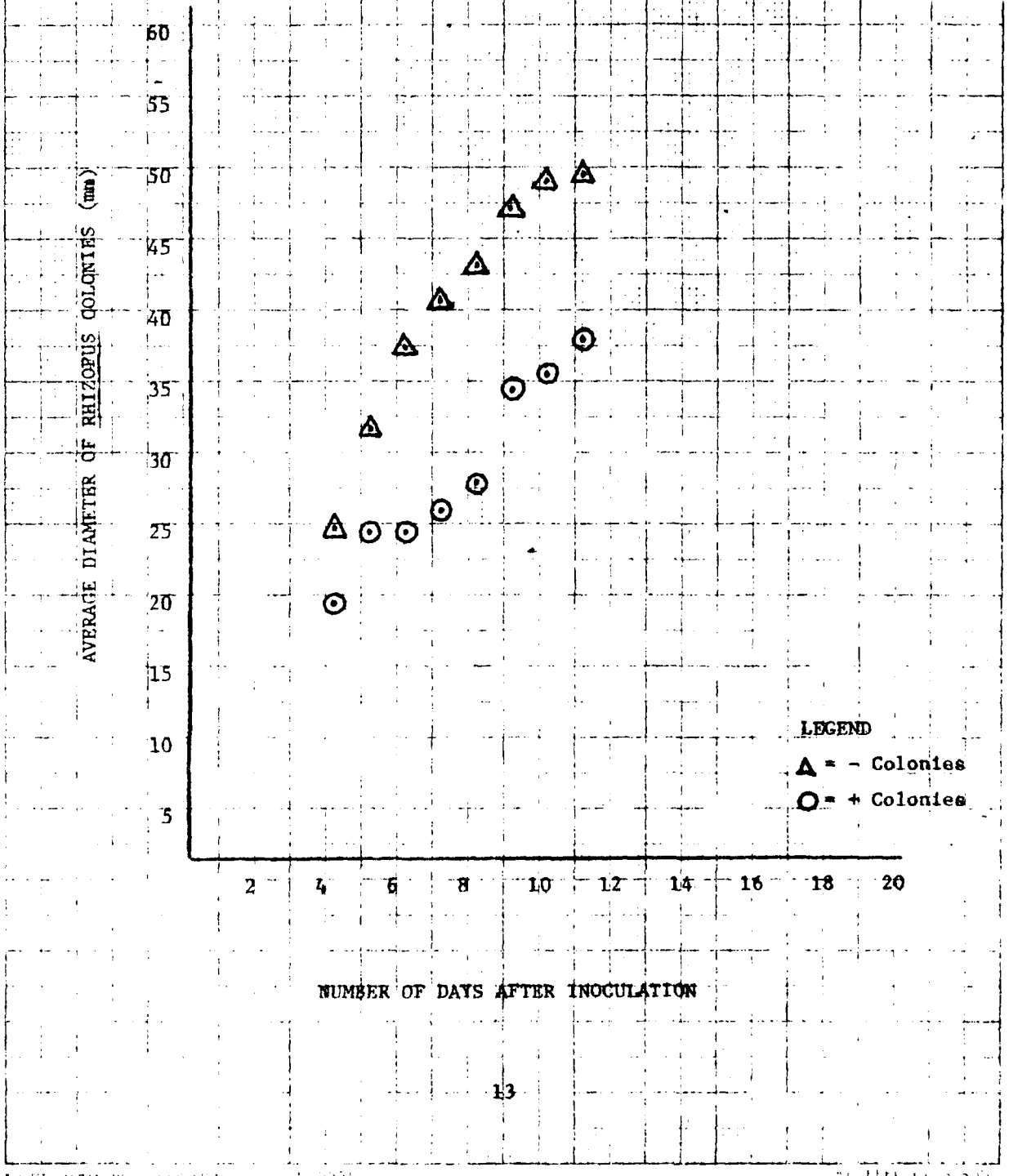


FIGURE 6

GROWTH CURVES FOR RHIZOPUS NIGRICANS CULTURES
SUBJECTED TO "STANDARD TREATMENT TABLE"
OXYGEN EXPOSURES (EXPERIMENT 2, RUN #2)

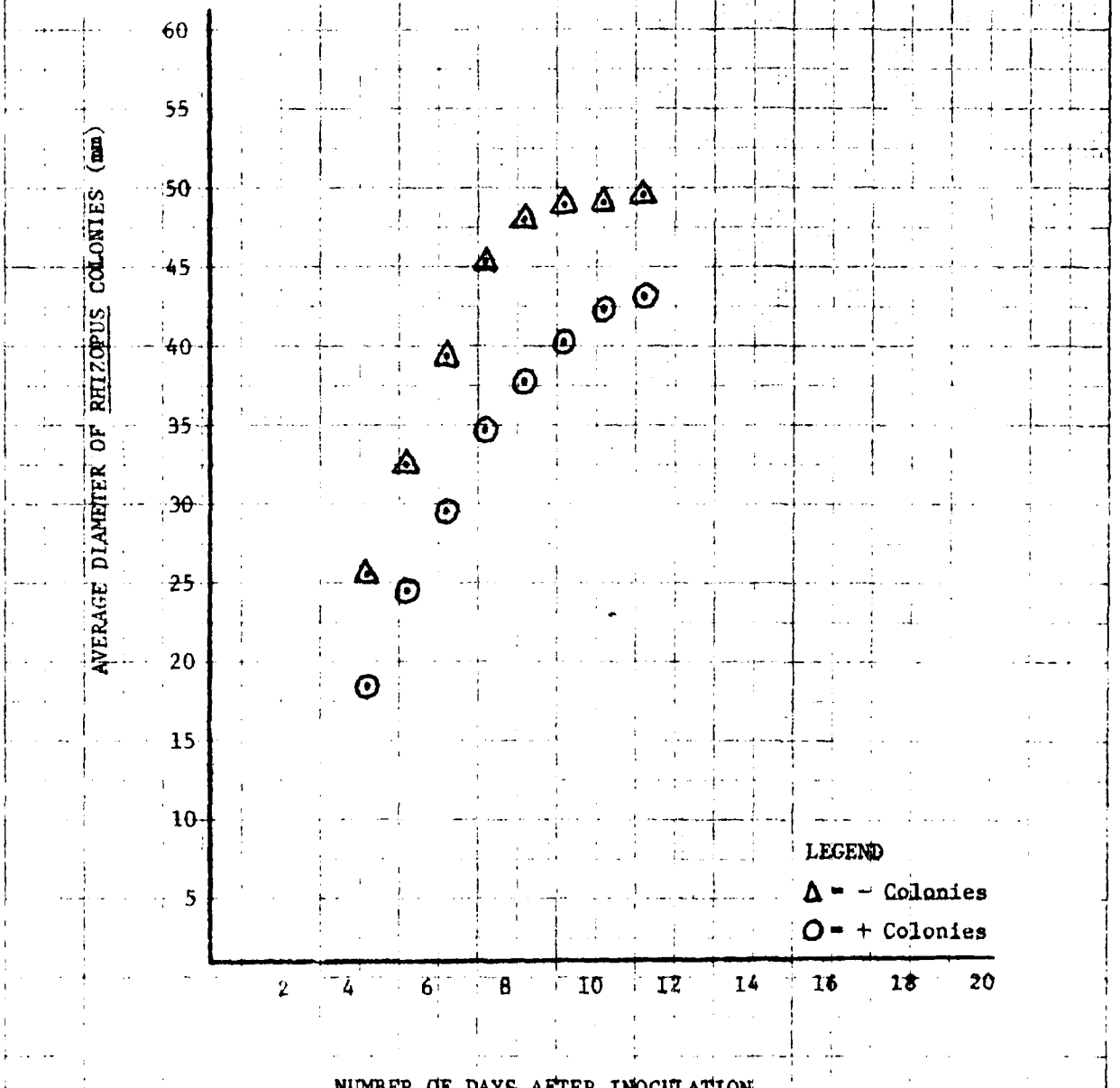


FIGURE 7

GROWTH CURVES FOR RHIZOPUS NIGRICANS CULTURES SUBJECTED TO "STANDARD TREATMENT TABLE" OXYGEN EXPOSURES PLUS TWO ADDITIONAL 90-MINUTE EXPOSURES AT 3-ATA

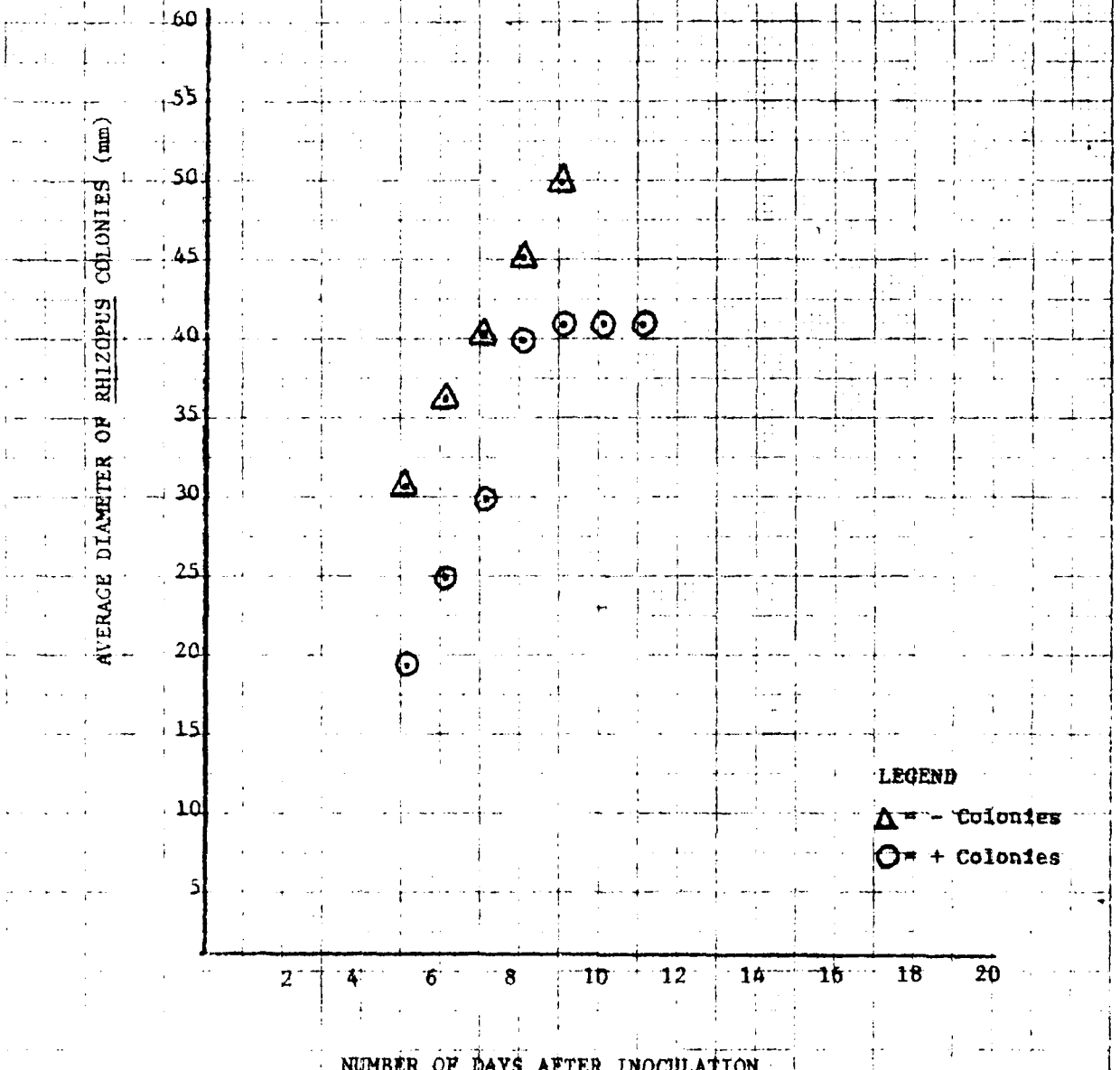
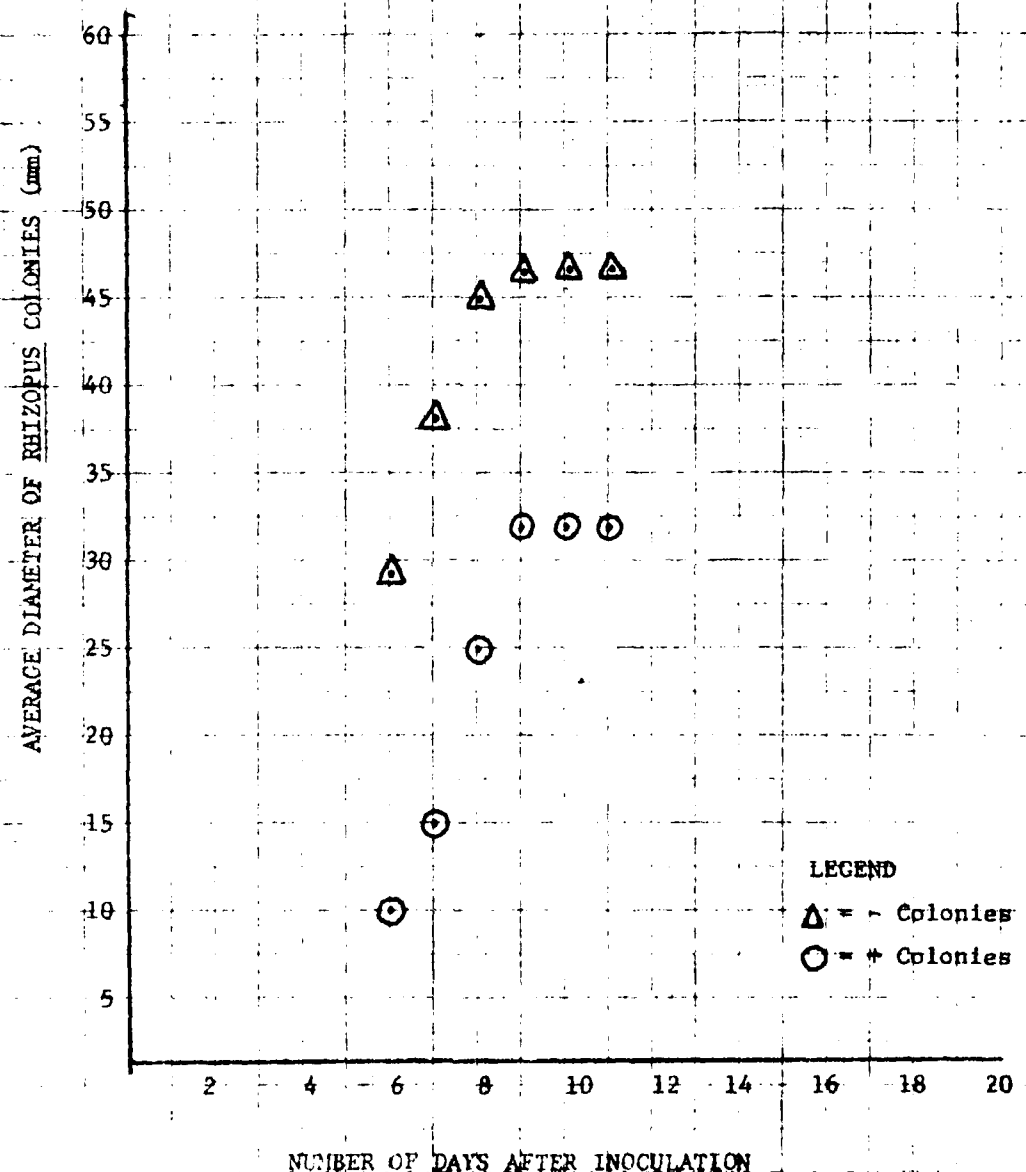
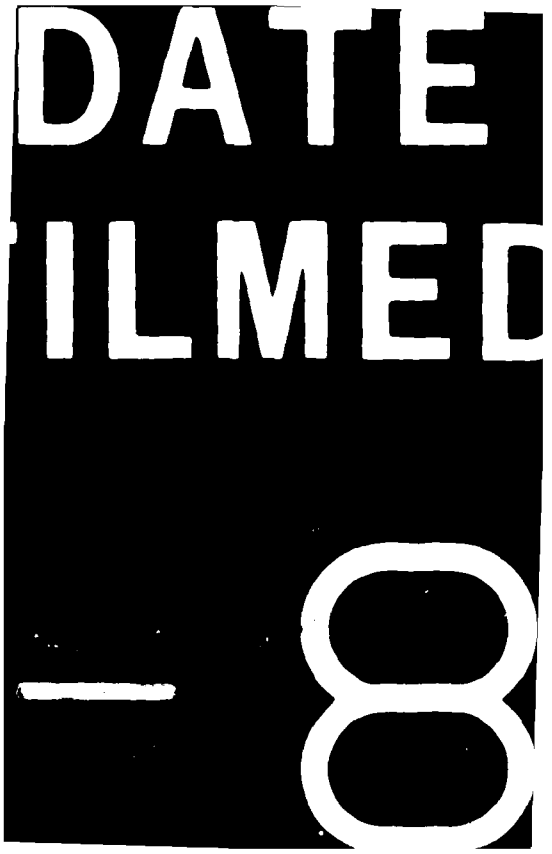


FIGURE 8
GROWTH CURVES FOR RHIZOPUS NIGRICANS CULTURES SUBJECTED TO "STANDARD' TREATMENT TABLE" OXYGEN EXPOSURES PLUS FOUR ADDITIONAL 90-MINUTE EXPOSURES AT 3-ATA

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